Mutation Research, 164 (1986) 3–8
Elsevier

MTR 08603

(1)

Feb,

Meeting Report

Mutagenesis and carcinogenesis by nitropyrenes and cancer chemotherapeutics

Frederick J. de Serres * and Taijiro Matsushima

12th Joint Conference of the U.S.–Japan Panel on Environmental Mutagenesis and Carcinogenesis, U.S.–Japan Cooperative Medical Sciences Program, Shimoda, Japan, 8–10 January 1985

(Received 18 September 1985)
(Accepted 14 October 1985)

The 12th Joint Conference on the Nitropyrenes and Cancer Chemotherapeutics was attended by 8 scientists from the United States where Dr. Frederick J. de Serres is Panel Chairman and 26 Scientists from Japan under the Chairmanship of Dr. Takeo Suzuki. A total of 4 sessions were held under the topics (1) Mutagenicity of Nitropyrenes, (2) Carcinogenicity of Nitropyrenes, (3) Secondary Cancer in Chemotherapy Patients and (4) Genetic Toxicology Studies in Cancer Chemotherapy.

Session I. Mutagenicity of Nitropyrenes

Dr. Yoshinari Ohnishi, The University of Tokushima, Tokushima, reported on the measurement of the mutagenicity of complex environmental mixtures and foods and the determination of the amounts of nitropyrenes in them. Nitropyrenes were found in the neutral and acidic fractions of exhaust particles from a diesel truck, in the benzene–ethanol (4 : 1) extract from the particulate fraction collected on Teflon-coated filters from a radiant kerosene heater and in the neutral fraction of sauce-coated, grilled chicken. The mutagenicity of these fractions was assayed with Salmonella strain TA98 in the absence of S9 mix. Furthermore, the carcinogenicity was examined by injecting 150 μg of 1,6-dinitropyrene mixed with bees wax and tricaprylin as a pellet into the lungs of F344 rats. The incidence of lung carcinomas was 23 out of 28 rats at week 72. In addition using germ-free and conventional rats, the metabolism of tritium labelled 1-nitropyrene was studied. The mutagenic activity of the feces and urine was studied using TA98 both with and without S9 mix and metabolites were identified using reversed phase HPLC after treatment with β-glucuronidase and their identity was confirmed by coelution with reference standards, by their UV spectra and by their mass spectra. These results suggest that the intestinal microflora reduces nitropyrenes to amino derivatives and that hydroxylation, conjugation and probably *N*-acetylation occur in the liver.

Dr. Robert Mermelstein, Xerox Corporation, Rochester, NY, reported on the extensive research on the genetic toxicology of nitrated pyrenes resulting from the discovery of potent bacterial mutagenicity and their widespread low-level dissemination. Nitropyrenes are potent, direct-acting mutagens for Salmonella and the pattern of activity indicates that frameshift mutations are being induced. The potency of individual nitropyrenes also differs as a function of tester strain. For example, 1,3-dinitropyrene and 1,3,6-trinitropyrene display the highest mutagenicity in Salmonella strain TA1538, 1,8-dinitropyrene is most active in strain TA98. There is substantial evidence for the mutagenicity of nitropyrenes in cultured mammalian cells: Chinese hamster ovary fibroblasts, Chinese hamster lung fibroblasts, hu-

* Address reprint requests to: Dr. Frederick J. de Serres, Office of the Director, National Institute of Environmental Health Sciences, P.O. Box 12233, Research Triangle Park, NC 27709 (U.S.A.).

0165-1161/86/$03.50

man hepatoma derived cell line Hep G-2 and human diploid lymphoblasts; however they are inactive in a human XP cell line. Various nitropyrenes have also been shown to stimulate DNA repair synthesis in HeLa cells, in primary rat hepatocytes as well as in Hep G-2 cells. Both 1-nitropyrene and 1,8-dinitropyrene have been shown to induce chromosome aberrations. Nitropyrenes exhibit a wide spectrum of genotoxic effects which are evident in both microbial and mammalian systems. The nature and extent of such activity depend on the metabolic capability of the individual test systems.

Dr. Hiroshi Tokiwa, Fukuoka Environmental Research Center, Dazaifu, discussed various sources of nitropyrenes in the environment and speculated that the increased use of radiant kerosene heaters and gas and liquified petroleum gas burners since 1955 may have been responsible for a concomitant increase in lung cancer in Japan. The emission soot from such burners contains mutagenic activity caused by dinitropyrenes and nitrofluoranthenes. When the gaseous fuel was burned under conditions of incomplete combustion, some polycyclic aromatic hydrocarbons (PAH) were found in addition to the nitropyrenes. In these combustion products of fuels, the quantities of 1-nitropyrene were unexpectedly low in contrast with those found in diesel emission.

Dr. Minako Naguo, National Cancer Center Research Institute, Tokyo, reported on the relative mutagenicity of 1,8-dinitropyrene (1,8-DNP) in Salmonella and in Chinese hamster lung cells in culture. In Salmonella, 1,8-DNP gives 9.4×10^4 revertants/μg with strain TA98 and 1.2×10^5 revertants/μg with strain TA100, but only 5.2×10^{-2} diphtheria toxin resistant mutants per 10 survivors/μg (−S9 mix).

Oncogenes of 8 fibrosarcomas induced by subcutaneous injection of 40 mg of 1,8-DNP were examined using NIH 3T3 cell transfection assay and Southern blot analysis. 4 tumors induced transformed foci in NIH 3T3 cells by DNA-mediated gene transfer. One primary transformant and its 7 secondary transformants contained active rat c-K-*ras*. The transforming gene of 6 other primary transformants obtained from 3 tumors were not *ras* family or *neu* genes.

Dr. Herbert S. Rosenkranz, Case Western Reserve University, Cleveland, OH, discussed the need to be able to predict the activity of, as yet, untested nitroarenes in the environment based on the properties of those nitroarenes whose properties have already been studied. Two different approaches are being used: (1) Electrochemical reduction of nitroarenes and (2) Computer Automated Structure Evaluation (CASE) Methodology. Electrochemical reduction can be used as an indication of the ease of reduction of the nitro function of the nitroarenes. A relationship was found between the ease of nitro-reduction ($E1/2$, the voltage of the half-wave potential) and the mutagenicity of these compounds in Salmonella assay using strains TA98 and TA1538. The $E1/2$ values were used to calculate the energy of the lowest unoccupied molecular orbital (LUMO energy) and this established that the mutagenicity of nitroarenes is directly related to LUMO. Since LUMO energies can be directly calculated, it is feasible to predict the mutagenicity of untested nitroarenes on the basis of the calculated LUMO energies. Using the CASE method, a mechanism has been developed for predicting the activity of untested nitroarenes based upon the presence of certain automatically determined structures. This CASE system has identified two activating and two deactivating substructures which explain the mutagenicity of nitroarenes. This method can also be used to investigate whether the functionalities responsible for mutagenicity and carcinogenicity are indeed identical.

Session II. Carcinogenicity of Nitropyrenes

Dr. Charles M. King, Michigan Cancer Foundation, Detroit, MI, reported on that induction of rat mammary gland tumors with various nitropyrenes. The initial evaluation of the carcinogenic potential of 1-nitropyrene employed both male and female CD rats. Treatment by subcutaneous injection was given from birth through approximately 60 days of age to provide exposure adequate for the development of either liver or mammary tumors. Tumor formation at the suprascapular site of injection was observed in both sexes at approximately the same frequency. Malignant mammary tumor induction was observed in a dose-dependent fash-

ion in the females. No evidence of liver tumor formation was detected. The results of this initial study was published in Cancer Res., 44 (1984) 1158–1162. In a second experiment, 1-nitropyrene, 2-nitropyrene, 4-nitropyrene, 1-acetylaminopyrene, *N*-hydroxy-1-acetylaminopyrene, *N*-hydroxy-2-acetylaminofluorene or DMSO alone were administered intraperitoneally to 30-day-old female rats. Mammory tumor induction was observed only with the 4-nitropyrene and *N*-hydroxy-2-acetylaminofluorene. Evidence of neoplastic nodule formation in the liver was observed only with the 3 acetylated derivatives.

Dr. Hiroko Ohgaki, National Cancer Center Research Institute, Tokyo, reported on experiments with highly purified samples of 1-nitropyrene (NP) and the 1,3-, 1,6- and 1,8-dinitropyrenes (DNP) to evaluate their carcinogenicity after subcutaneous injection in F344 rats. Test chemicals dissolved in 0.2 ml DMSO were injected twice a week for 10 weeks. The three dinitropyrenes were found to produce subcutaneous tumors at the site of injection in 10/10 rats injected with a total of 4 mg of the test chemical.

Dose-response relations were studied by injecting F344 rats subcutaneously with 4, 0.4 or 0.04 mg of 1,8-DNP. Sarcomas at the site of injection were found in 10/10 animals by 127 days, 10/10 animals by 156 days 9/10 animals by 320 days, respectively, for each of the three treatments. Previous studies with 1-nitropyrene (Cancer Lett., 15 (1982) 1) showed that it produced subcutaneous tumors at the site of injection in 8/17 rats between 162 and 319 days. However, this batch of 1-nitropyrene was later found to be contaminated with 0.2% 1,3-DNP, 0.3% 1,6-DNP and 0.3% 1,8-DNP raising the possibility that the carcinogenicity was due to the dinitropyrene contaminants. The 1-NP experiment was repeated using a highly purified sample where the DNP level was < 0.05%, 1,3,6-trinitropyrene < 0.05% and 1,3,6,8-tetranitropyrene < 0.05%. 10 and 20 male F344 rats were each injected subcutaneously 2 times for 10 weeks to give a total dose of 4 or 40 mg 1-NP. No tumors were found by day 650 clearly demonstrating that the previous positive carcinogenicity test with 1-NP was due to the trace levels of DNP contaminants.

Dr. Shozo Takayama, National Cancer Center Research Institute, Tokyo (paper presented in his absence by Dr. Shigeaki Sato) reported on recent experiments to study the induction of lung cancer by 1,6-dinitropyrene. The 1,6-DNP was ground and suspended at a concentration of 2.5 mg/ml and a long needle was used to give a tracheal dose of 0.5 mg once a week for 26 weeks into 10-week-old female (10) and male (10) Syrian golden hamsters. All treated, and a similar number of saline-treated control animals, were observed for 48 weeks after the first treatment. Lung tumors started to appear in the female 1,6-DNP-treated animals after 20 weeks and many additional tumors appeared during the next 20 weeks in both male and female animals. Lung adenocarcinomas were found in 10/10 males and 9/10 females, and myeloid leukemias were also induced in 6/10 males and 6/10 females. No tumors were found in the control animals.

Dr. Hiroshi Tokiwa, Fukuoka Environmental Research Center, Dazaifu, reported briefly on some experiments to evaluate the carcinogenic activity of a sample of 99.9% pure 1,6-DNP in BALB/c mice. Using total doses of 2 mg given subcutaneously with 0.1 mg/week for 20 weeks a comparison was made between pure 1-NP and 1,6-DNP. No tumors were found with 1-NP, malignant fibrous histocytomas at the site of injection were found with 1,6-DNP and the incidence of lung tumors did not differ significantly from that found in the control animals in either treated group. Similar tumors in the 1,8-DNP-treated mice were found at the high rate when 1 mg of 1,8-DNP was inoculated subcutaneously.

Dr. Kazuro Iwai, Japan Anti-tuberculosis Association, Tokyo, reported on chronic inhalation experiments to investigate the tumorgenicity of diesel exhaust particles in F344SPF rats. Proliferative changes of bronchiolar and alveolar epitheliums were found in the early stage and both benign and malignant lung tumors were induced in the late stage.

Session III. Secondary Cancer in Chemotherapy Patients

Dr. Shaw Watanabe, National Cancer Center Research Institute, Tokyo, reported on his studies on second primary malignancies in long-term survivors of cancer chemotherapy. Patients who received alkylating agent therapy for cancer of the ovary, breast, lung, brain and myeloma have also been shown to be at increased risk for developing acute leukemias. Data collected by Fujimoto in Osaka on 31 cases with secondary heterochronous leukemia were analyzed by matched case-control study. The cases with leukemias revealed a high occurrence of leukemias in chemotherapy and radiation and less in the surgery only group. In a hospital based study performed at the National Cancer Center, 270 multiple primary cancer (MPC) were found among 9191 patients initially treated for GI tract cancer, 94 MPC among 1967 initial lung cancer patients, 62 MPC among 2715 initial breast cancer patiens and 68 MPC among 2682 initial uterine cervical cancer.

Dr. Leslie L. Robison, University of Minnesota Health Sciences Center, Minneapolis, MN, discussed the occurrence of secondary tumors in the survivors of childhood cancer. During the past 20 years significant advances have been made in the treatment of childhood malignancies. Approximate 5-year survival rates are: acute lymphoblastic leukemia (65%), acute nonlymphoblastic leukemia (40%), brain tumors (40%), Wilms' tumor (90%), Hodgkin lymphoma (90%), neuroblastoma (30%) and retinoblastoma (85%). Second malignant neoplasms (SMN) represent one of the most serious complications for survivors of childhood cancer. The incidence of SMN in persons with cancer in childhood has been estimated to be between 10–15% at 25 years following the diagnosis of the initial malignancy. Most frequently reported SMN were in patients with first tumors of retinoblastoma (18%), Hodgkin disease (14%), soft tissue sarcomas (14%), Wilms' tumor (12%) brain tumors (11%), neuroblastoma (10%), bone sarcomas (6%), and leukemia or non-Hodgkin lymphoma (8%). Studies conducted by the Late Effects Study Group in conjunction with the National Cancer Institute have assessed the risk of SMN associated with specific groups of chemotherapeutic agents. After adjusting for radiation exposure, alkylating agents were found to be significantly associated with an increased risk for leukemia. A significant dose response was identified with highest levels of alkylating agent exposure denoting a 23-fold increased risk for leukemia. In addition, a 2-fold risk for bone cancer as a second malignancy was found for alkylating agent exposure independent of radiation exposure. Well-designed studies of large cohorts of children diagnosed and treated for cancer will be needed to accurately assess the risk of SMN and to provide insight into the genetic and treatment components of the development of SMN.

Session IV. Genetic Toxicity Studies in Cancer Chemotherapy

Dr. John Mulvihill, National Cancer Institute, Bethesda, MD, reported on studies of cancer and genetic disease in progeny of cancer patients. Cancer treatment is often designed to interact specifically with DNA and to block its function. Cancer therapy can cause somatic cell mutation, as evidenced by the excessive risk of additional primary malignant neoplasms and other measures of genetic damage including chromosome abérrations. Cancer and its treatment may also cause infertility. If a pregnancy is established in or by a cancer patient, the outcome is of interest: pregnancies occurring *before* cancer diagnosis serve as a control experience and a measure of the heritable predisposition to cancer (or to other genetic diseases). Pregnancy occurring *during* cancer therapy can be evaluated for teratogenicity and embryotoxicity. Pregnancies *after* cancer therapy reflect survival of gonads and intact reproductive tracts as well as potential germ cell mutagenicity. In 9 large studies with over 467 patients and 993 complete pregnancies, only 32 (4%) of 747 live-borns had one or more major birth defects, a rate comparable to that found in the general population. None was a classical sentinel phenotype. A new study of survivors of childhood cancer focused on the occurrence of new primary cancers, late morbidity, and infertility in the cases, and cancer and birth defects in their offspring. Patients were studied for secondary malignancies among those

children who had cancer under the age of 20 between 1945 and 1974, and who had survived at least five years and reached the age of 21 years. 97 subsequent tumors occurred in 2293 cancer cases. 8 of 2328 offspring of cases had cancer and 10 of 4789 offspring of controls. One or more major birth defects occurred in 192 (8%) of case children and 353 (7.4%) of control children. These rates are not statistically different.

Dr. Michael D. Shelby, National Institute of Environmental Health Sciences, Research Triangle Park, NC, reported on studies to determine the frequencies of chromosome aberrations and sister-chromatid exchange in control populations. The measurement of chromosomal damage in peripheral lymphocytes offers a practical means of detecting exposure of humans to genotoxic agents. Comparisons of results obtained with different groups of exposed individuals is often difficult due to differences in protocols. Thus, agreement on a basic protocol for human cytogenetic studies is needed, as is information on the background frequencies of chromosomal aberrations (CA), particularly chromatid-type aberrations, and sister-chromatid exchanges (SCE), their variability and the sources of variability. Such information cannot take the place of concurrent controls but will be useful in the design and interpretation of future cytogenetic studies of populations exposed to potential chromosome-damaging agents. In work carried out at the Oak Ridge National Laboratory and at the Brookhaven National Laboratory under NIEHS/NTP contract, CA and SCE data have been obtained on 357 subjects not known to be exposed to clastogenic agents, the majority selected using a stratified, random sampling scheme. In comparisons of chromosome aberration data using the dichotomous variables: sex, smoking, work with solvents, work with radiation and use of prescription drugs, no significant differences were seen. SCE frequencies, which average 8.49 per cell with a range of 5.70–14.98, were significantly higher in smokers than in non-smokers. Preliminary analyses have not shown the other variables to influence SCE frequencies.

Dr. Yutaka Ishii, Osaka University, Osaka, reported on his studies to evaluate the genetic effects of various chemotherapeutic agents by measuring Sister-Chromatid Exchange (SCE) frequencies in peripheral blood lymphocytes from cancer patients. The agents studied were mitomycin C (MC) and cyclophosphamide (CP). In the MC experiments the drug was injected intraveneously twice a week for 2 weeks in 2 patients with gastric cancer. After the first and second treatment with MC, SCE frequency increased with time, reached a peak after 24 h, and then declined returning to the pretreatment level at about 2 days following treatment. After the third and fourth course of injections, SCE frequency increased further and did not return to the pretreatment level by 3 days after treatment. However SCE frequencies were at pretreatment levels by 3 months after treatment. A similar experiment was performed with CP. After injection of the drug SCE frequencies would increase rapidly with time, reaching a peak in 24 h and returning to pretreatment levels on the 7th day. Similar cycles of rapid increase and slow decrease in SCE frequency were obtained after each of the subsequent 5 injections, resulting in a gradual rise in the basal and the maximum level of SCE. In the 5th month after termination of therapy, SCE frequency returned to the pretreatment level. Thus, SCE frequency may quantitatively reflect the degree of genomic damage by a chemotherapeutic agent at the time of administration but it may not be a good index of genomic damage persisting for a long time after therapy.

Dr. Mortimer Mendelsohn, Lawrence Livermore National Laboratory, Livermore, reported on newly developing assays as LLNL to measure somatic and germinal genetic damage in the human. The first project involves the development of an assay for gene loss in human red blood cells. Highly specific fluorescent monoclonal antibodies against the products of the M and N alleles of glycophorin A have been developed and can be differentially measured (red fluorescence for the N allele and green fluorescence for the M allele) on the red cell surface by flow cytometry. MN individuals constitute about 50% of the human population. The two alleles act independently so if one or another is inactivated the glycophorin A made is produced only by the active allele. Experiments are in progress to measure the changes in frequency

in the red blood cells from cancer chemotherapy patients. In another line of work hemoglobin assays for mutations resulting in single amino acid changes in human, monkey and mouse red blood cells are in progress. The previously available polyclonal immunological assay for human hemoglobin S is now supplanted by a monoclonal approach and is supplemented by a variety of other antibodies which also recognize the effects in the hemoglobins of several species of single-base changes in the DNA of precursor cells. New work has been started to develop a monoclonal antibody against protamines (histone-like proteins in the sperm) made in the late stages of spermatogenesis. The protamines have been sequenced to determine their normal amino acid composition, but there still is a question as to whether mutations that would alter this sequence would be compatible with normal spermatogenesis. In another project antibodies to bromodeoxyuridine (BrDR) permit testing of the effect of the BuDR on the production of sister-chromatid exchanges (SCE) at very low doses. These studies have shown that BuDR does cause SCE, but at very low levels a plateau is reached which may reflect the normal spontaneous SCE frequency. New experiments with flow-cytogenetic methods for karyotyping have been used in studies with the Chinese hamster. By measuring the DNA fluorescence for chromosomes at metaphase all chromosomes can be characterized. Similar studies have been done with human chromosomes using the Hoechst stain or the chromomycin A stain. Different chromosomes map in a characteristic place. When fluorescence is mapped against DNA content; chromosomes 9-12 form a single elipse and chromosomes 14 and 15 overlap but all other chromosomes are separate from one another. Flow karyotyping can now be used to determine whether the karotype is normal or abnormal and can be used in combination with amniocentesis for prenatal diagnosis. This same technology can be used to get a profile of DNA along the length of chromosomes and thus to identify centromere location and dicentrics. The sorting of human chromosomes leads to the construction of gene libraries of specific chromosomes. A total of about 4×10^6 chromosomes of a particular chromosome in the genome can be obtained with this methodology and the DNA extracted. In a joint project with the Los Alamos National Laboratory, half of the human genome has now been cloned, chromosome by chromosome. In other lines of work recombinant DNA markers for individual human chromosomes are being developed. By using fluorescent markers these chromosome specific DNA probes can be used to stain interphase cells to study the spatial array of the DNA in individual chromosomes during this stage of the cell cycle that has been refractory to conventional methods of cytological analysis. The final project involves study of the cytogenetics of mature human sperm. This work is possible because the hamster oocyte will accept human sperm. When colchicine is used to arrest cell division in the early stages of development of the fertilized egg, the haploid human sperm set of chromosomes can be analyzed and karyotyped. A total of 2468 karyotypes have been studied; 181 (7.5%) have structural abnormalities; 21 (0.9%) are hyperploid and 29 (1.6%) are hypoploid. The abnormality rates for individuals donors seem to be stable with time suggesting that this assay provides a true measure of genetic damage. However, sperm karyotypes consistently yielded higher aberration frequencies in the same individual than is obtained with lymphocytes in culture; the basis for this difference has not as yet been determined. This technique does provide a mechanism to evaluate the genetic effects of exposure to mutagenic/carcinogenic agents and would be especially useful in those cases where a karyotype analysis has been made prior to such exposure so that each individual can serve as his own control.